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Analysis of Fetotoxicity Using Biochemical Endpoints of Organ Differentiation

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An Analysis of Fetotoxicity Using Biochemical Endpoints of Organ Differentiation

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ABSTRACT The biochemical differentiation of the brain, lungs, liver, and kidneys of the late gestation rat fetus was examined to characterize the immediate implications of retarded growth on fetal development. Initially, the normative profile of development of the brain (weight, DNA content, and protein content), lungs (weight and surfactant accumulation), liver (weight and glycogen deposition), and kidneys (weight, alkaline phosphatase activity, and protein content) was determined on gestation days 19, 20, 21, and 22 (day 1 = finding of sperm in the vaginal smear). Subsequently, five compounds known to induce fetotoxicity (chlorambucil, methyl salicylate, mirex, nitrofen, and toxaphene) were administered during organogenesis, and the effects on organ differentiation were determined in day 21 fetuses. The effects of fetal growth retardation resulting from exposure to exogenous agents were not equally distributed among the organs studied. The liver and kidney appeared more sensitive to insult by these agents than did the brain and lungs.

Results of standard teratology bioassays include: 1) death of the conceptus, 2) the occurrence of frank terata, 3) increased incidence of so-called minor anomalies (e.g., supernumerary and/or wavy ribs, delayed ossification of the skeleton, enlarged renal pelvis, and enlarged cerebral ventricles), 4) retardation in overall growth patterns, and 5) no observable effect. The interpretation of the first two items is relatively straightforward: the compound is considered a potential human teratogen, the dose-response relationship is determined empirically, and a decision regarding usage patterns is made using risk-benefit analyses. At the other end of the spectrum, compounds that produce no observable effects in standard teratology bioassays are considered to have a low potential to induce terata in humans and are allowed wider usage patterns. Between these two extremes, the interpretation of the results becomes considerably more complex. Fetal growth retardation and altered incidences of minor anomalies (generally referred to as "fetotoxicity") may be observed at dose levels below those which are overtly teratogenic or they may be accompanied by evidence of maternal toxicity with subsequent confounding of cause and effect determinations. The biological significance of the fetotoxic responses is poorly understood. Do they merely represent developmental delays that attenuate with time or do they foreshadow more serious forms of toxicity? This question can only be answered after the relationship(s) between fetotoxic effects and potential postnatal health effects are elucidated.

This study was undertaken to characterize the immediate implications of fetotoxicity in terms of the growth and biochemical differentiation of key organ systems in the late gestation rat fetus. The biochemical end points examined were selected because of their relevance to perinatal organ function and as such represented reliable and sensitive indicators of fetal maturation. Specifically, we examined the brain for DNA and protein content (indicators of cell number and cell size, [Zamenhof et al., '64]), the lung for phosphatidylcholine and sphingomyelin content (the primary surface active materials [Perelman et al., '81]), the liver for glycogen deposition (a vital energy source for the newborn pup [Jacquot et al.,

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'73]), and the kidney for protein content and alkaline phosphatase activity (a marker of renal tubular development [Wachstein, '55]). After constructing normative profiles for these variables during late gestation, we examined the effects of five chemicals known to induce fetotoxicity on the status of organ differentiation in 21-day-old fetuses. Once the significance of fetotoxicity on the development of specific organs is better understood, studies can then be designed to examine whether or not developmental alterations persist postnatally as impaired organ function.

MATERIALS AND METHODS

Animals

Time pregnant, 90-day-old CD rats were obtained from Charles River Breeding Laboratories, Wilmington, Massachusetts. Day 1 of pregnancy was recorded upon demonstration of sperm in the vaginal smear. Rats were housed individually in polyethylene cages on pine shaving bedding and allowed Purina Lab Chow and water ad libitum. Animal rooms were maintained at constant temperature (20–24 °C) with controlled photoperiod (12 hour light).

Tissue collection

At the designated gestational age, dams received an intraperitoneal injection of sodium pentobarbital (36 mg/kg). A laparotomy was performed, and five fetuses were removed sequentially from the left uterine horn beginning at the ovarian end. Immediately upon removal from the uterus, the fetuses were weighed and decapitated. The brain (upper medulla, cerebellum, and cerebrum exclusive of the olfactory lobes), lungs, liver, and kidneys were dissected out, weighed to the nearest milligram quick frozen in an acetone/dry ice bath, placed in polyethylene vials, and stored at −60 °C until the biochemical assays were carried out. The lungs were rinsed briefly in isotonic saline prior to freezing in order to remove blood.

Biochemical assays

DNA and protein were extracted from the brain as described by Margolis ('69) with the exception that the tissue was homogenized using a Brinkmann Polytron. DNA content was determined by the diphenylamine procedure of Burton ('56) with the modification in absorbance readings used by Margolis ('69). Calf thymus DNA (Sigma Chemical Co.) was used as the standard. Protein pellets were suspended in 8 ml of 0.3 N NaOH and the protein content was determined by the Peterson modification of the Lowry technique (Peterson, '77) using bovine serum albumin (Sigma Chemical Co.) as the standard.

Lungs were processed in a manner similar to that described by Daston Grabowski ('79). Tissues were homogenized in 3 ml of chloroform/methanol (2:1) and centrifuged at 5,000g for 15 minutes. The supernatant was rinsed with 1 ml of 0.74% KCl and evaporated to dryness at 40 °C under nitrogen. A sufficient quantity of chloroform/methanol (1:1) was added to solubilize the phospholipids, followed by 5 ml of 5 °C acetone. This preparation was kept at 5 °C for 3 hours and then centrifuged at 5,000g for 15 minutes. The supernatant was discarded and the pellet of surface active phospholipids solubilized with 1:1 chloroform/methanol (9 μl/mg lung). Duplicate 20 μl aliquots were spotted (TLC Multi-Spotter, Analytical Instrument Specialties) on heat activated Silica Gel GF 10 × 20 cm glass plates (Analtech, Inc.). Plates were developed using a solvent system of chloroform/methanol/ammonium hydroxide/water (70:30:2:4) (Colacicco et al., '77). Known quantities of dipalmitoyl phosphatidylcholine and sphingomyelin (Sigma Chemical Co.) were spotted on each plate. Visualization of the phospholipids was achieved by spraying the plate to saturation with phosphomolybdic acid (10% in ethanol) followed by heating at 120 °C for 45 minutes. Plates were scanned by reflectance densitometry (SD 3000 Spectrodensitometer, Schoeffel Instrument Co., Inc.) and peak areas determined by integration (Shimadzu C-RIA Chromatopac).

Liver glycogen was extracted and assayed by the anthrone technique of Carroll et al., ('55) with the exceptions that tissues were homogenized using a Brinkmann Polytron, and centrifugations were performed at 10,000g.

Kidneys were homogenized in 1 ml of pH 7.4 HEPES buffer in 2 ml Potter-Elvehjem tissue homogenizer, centrifuged at 5,000g for 15 minutes, and assayed for alkaline phosphatase activity using Sigma 104 Phosphatase Substrate (Sigma Chemical Co.) and for protein content by the Peterson modification of the Lowry technique (Peterson, '77).

Normative series

To construct the ontogenetic profile of the selected variables and to provide a data set with which to compare subsequent treatment-induced alterations, six dams were killed on each of gestational days 19, 20, 21, and 22, and fetuses and organs examined as described above.

Experimental series

Five compounds were selected from the literature on the basis of their reported ability to induce fetotoxicity. These included the nitrogen mustard chlorambucil (CHL), methyl salicylate (MES), the insecticides mirex (MIR) and toxaphene (TOX), and the herbicide nitrofen (NIT). Details of the treatment regimens are provided in Table 1. Administration of CHL followed the protocol of Monie ('61), MES that of Woo and Hoar ('72), MIR that of Chernoff et al. ('79), NIT that of Kimbrough et al. ('74) and TOX that of Chernoff and Carver ('76). Five or six dams from each dosage group were killed on gestational day 21 and the fetuses and organs processed as described above. Fetuses with readily identifiable external malformations were excluded from biochemical analysis. All fetuses not used in the biochemical analysis were fixed in a solution of 70% ethanol, 5% formaldehyde, and 5% glacial acetic acid and examined for external and internal abnormalities.

Statistics

All data were analyzed using procedures available on version 79.4B of the Statistical Analytical System (SAS, 1979). The litter was regarded as the experimental unit in all statistical comparisons. The normative series was analyzed by one way ANOVA using gestational age as the dependent variable. When this analysis was significant at the 0.05 level, individual means were compared using Duncan's Multiple Range Test. In the experimental series, treatment effects were analyzed by one-tailed linear regression analysis using fetal weight as a covariate (Snedecor and Cochran, '71). This analysis allowed us to determine if the observed organ toxicity was independent of, or correlated with, the overall growth retardation. When the overall anaysis was significant, the means (both with and without adjustments for differences in body weight) were compared using a one-tailed t-test. One-tailed tests were employed because it was our a priori hypothesis that reductions in organ growth or biochemical differentiation and not accelerations would occur as a result of treatment. While some aspects of developmental processes may be enhanced by exposure to certain chemicals (e.g., the ability of dioxin to cause premature appearance of hepatic drug metabolizing enzymes), we feel that due to the

TABLE 1. Treatment regimes and developmental parameters of selected compounds

Compound	Route	Gestational age of treatment	Dose (mg/kg/day)	No. litters	Avg. No. implants	Avg. fetal mortality (%)	Avg. fetal weight (g)	Soft tissue abnormalities[2]
Chlorambucil	IP	11	0	5	12±1[1]	15±7	3.99±0.10	
			3	5	10±1	2±4	3.79±0.10	
			6	6	12±1	28±15	3.07±0.20	DH 15/6, EG 5/4, IR 9/5, MS 1/1, OD 22/9, PD 1/1, RA 8/5
Methyl salicylate	IP	9-10	0	5	12±1	11±5	4.14±0.08	
			200	5	12±1	2±2	3.87±0.13	DH 1/1, EN 2/2
			400	5	9±1	50±5	2.71±0.36	CP 1/1, EN 2/1, GS 2/1, HC 1/1, SB 2/1
Mirex	PO	7-16	0	6	12±1	0±0	4.27±0.19	
			6	6	12±1	4±2	4.17±0.27	ED 16/4, EG 4/2, HC 1/1
			12	6	12±1	8±4	3.79±0.81	ED 33/6, EG 3/1, HC 1/1
Nitrofen	PO	8-16	0	5	12±1	2±2	4.20±0.16	
			12.5	5	13±1	5±4	3.77±0.20	DH 9/3
			25	5	12±1	8±4	3.49±0.17	DH 17/5, EG 1/1, IR 2/1
Toxaphene	PO	7-16	0	5	11±1	1±1	3.93±0.25	
			12.5	5	10±1	3±3	4.16±0.16	
			25	5	12±1	0±0	4.16±0.41	

[1]Data presented as mean ± standard error.
[2]Data presented as number fetuses affected/number litters affected. CP = cleft palate, DH = diaphragmatic hernia, ED = edema, EG = ectopic gonads, EN = encephalocele, GS = gastroschisis, HC = hydrocephaly, IR = irregular palatine rugae, MS = microstomatia, OD = oligodactyly, PD = polydactyly, RA = renal agenesis, SB = spina bifida.

facts that exposures occurred early in development, and the nature of the end points used in this study that such effects would be unlikely to occur and hence one-tailed statistical tests were appropriate. All effects are reported as significant at the $P < .05$ level.

To develop a model for estimating the developmental age of an organ following exposure to a chemical, factor analysis was applied to the data obtained in the normative series (see Miller et al., ['77] for a discussion of the utility of this approach in describing developmental processes). We feel that this type of analysis provides a useful tool for simplifying the descriptive presentation of the large amount of data contained in this study despite the fact it may distort actual developmental ages because of factors affecting organ weights, such as edema, hemorrhage, and fatty infiltration, that were not accounted for in the research methods. This analysis allowed us to describe the correlations between the measured variables and to determine if organ maturation was a single event or represented several independent events. Only one factor was identified for each organ system that explained a significant amount of variation (i.e., had an eigen value of greater than one). Therefore, when we applied the linear weighting coefficients obtained in the factor analyses to the organ parameters (weight plus related biochemical values) in the normative series, we obtained a single composite variable for each organ system. These composite variables showed highly significant linear relationships with fetal age (r^2 values of 0.83, 0.95, 0.83, and 0.85 were obtained for the brain, liver, lungs, and kidneys, respectively). We then applied these coefficients to the data from the experimental series to obtain a single value representative of the state of development of each organ. Finally, the composite organ indices were converted into estimates of the age of organ differentiation using the regression equations from the normative series. Similarly, fetal weight data in the normative series were regressed on age ($r^2 = 0.94$), and data from the experimental series were converted into age estimates of the fetuses based upon their weights. These theoretical ages allowed us to directly compare the magnitude of the effects on each organ versus the fetus as a whole.

RESULTS

Normative series

The morphological and biochemical growth of control fetuses over the last four days of gestation is presented in Figure 1. To best display the dynamic aspects of organ development, organ weight data is presented relative to the body weight and the biochemical variables as relative to the respective organ weight. During the observed period of development, fetal weight increased from 1.44 gm on day 19 to 5.89 gm on day 22 (with weights of 2.54 and 3.84 gm recorded on day 20, respectively). The brain and liver both decreased in size relative to the body as a whole from day 19 to 22, while the lung reached its peak relative to body weight on days 20 to 22, and the kidney showed a steady increase in relative weight. Growth of the brain occurred more through cell enlargement rather than cell division, as the relative amount of DNA decreased and the relative amount of protein remained constant during this time. Pulmonary phospholipids showed their expected surge in synthesis between days 21 and 22, as did the hepatic glycogen concentration. Similar to the pattern observed in the brain, renal protein content remained constant during development. Renal alkaline phosphatase activity increased dramatically between each observation period, with relative amounts on day 22 almost 14 times those present on day 19.

Experimental series

The effects of the five compounds on fetal organ development are presented in Table 1 (overall developmental parameters) and Figures 2–5 (for the brain, lungs, liver, and kidney, respectively). In addition, the composite indices of organ development derived from comparison with the normative series are presented in Figure 6. All compounds except TOX reduced fetal weight in dose-related fashion. Embryolethal effects were evident only in the high dose MES group. Teratogenic responses were noted in both dose groups for NIT and MES, and the high dose group for CHL, while edematous fetuses were observed in both MIR groups.

Fetal growth

The fetal body weight index (i.e., the theoretical gestational age of the fetus based upon its weight relative to the weights observed in the normative series) was significantly reduced in the high CHL group, the high MES group, the high MIR group, and in the low and high NIT groups. These development alterations in body growth ranged from a 0.36 day delay in the low NIT group to a 0.96 day delay in the high MES group.

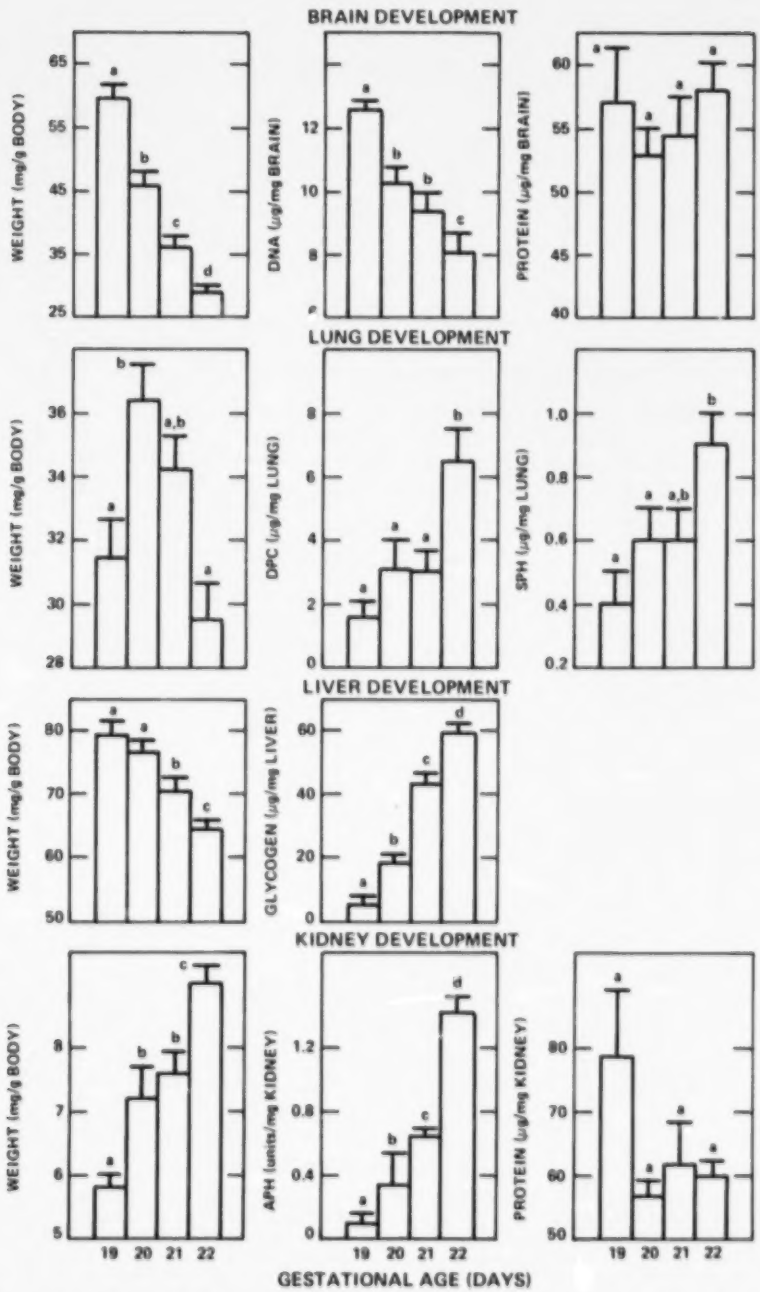

Fig. 1. Normative patterns of organ growth and biochemical differentiation in the late gestation rat fetus. Data are from five fetuses from each of six litters on gestation days 19, 20, 21, 22. Data presented as the litter means with standard error bars. Means with the same superscript are not significantly different at the $P<.05$ level (Duncan's Multiple Range test). DPC = dipalmitoyl phosphatidylcholine, SPH = sphingomyelin, APH = alkaline phosphatase activity.

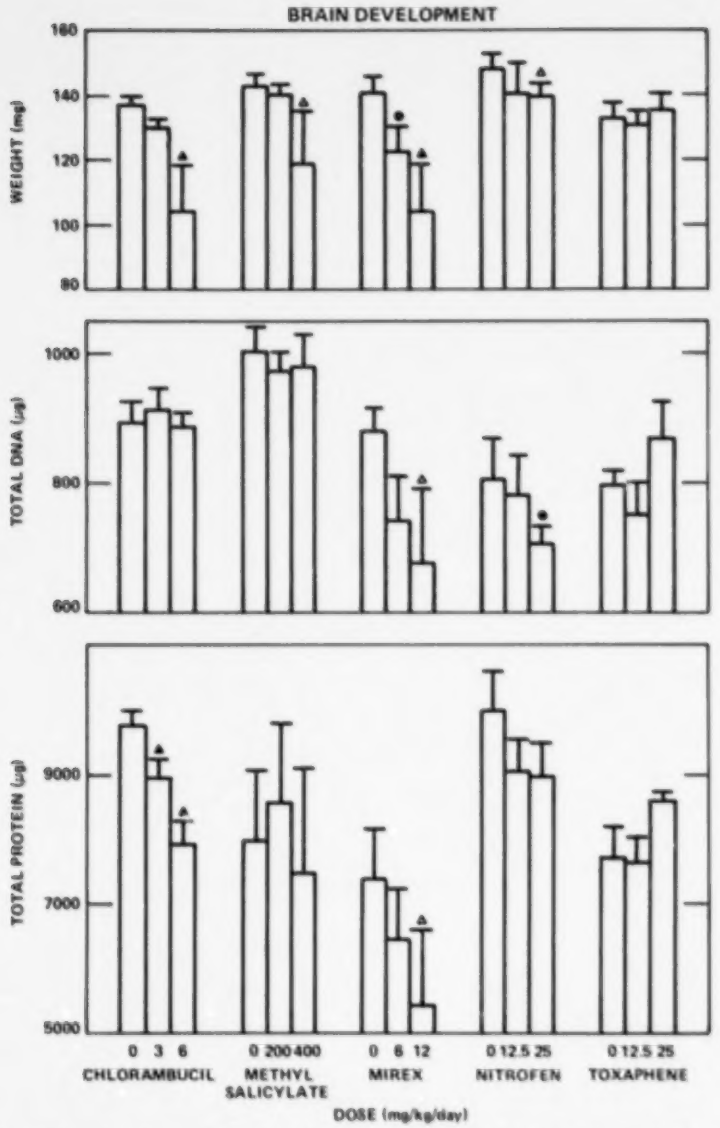

Fig. 2. Effects of five selected compounds on brain development in 21-day-old rat fetus. Data are from five pups from each of five to six litters for each dosage group. Data presented as the litter mean with standard error bars. When a significant dose-related reductic : n a variable was noted by linear regression analysis, means from the treated groups were compared with values for their concurrent control group using a Least Significant Difference test. The following symbols were used to represent significant differences between treated and concurrent control group means: △ indicates a significant difference between true means; ▲ indicates a difference between treated and concurrent control values both for true means and means adjusted for differences in body weight, and ● indicates differences between treated and concurrent control values only between means adjusted for differences in body weight.

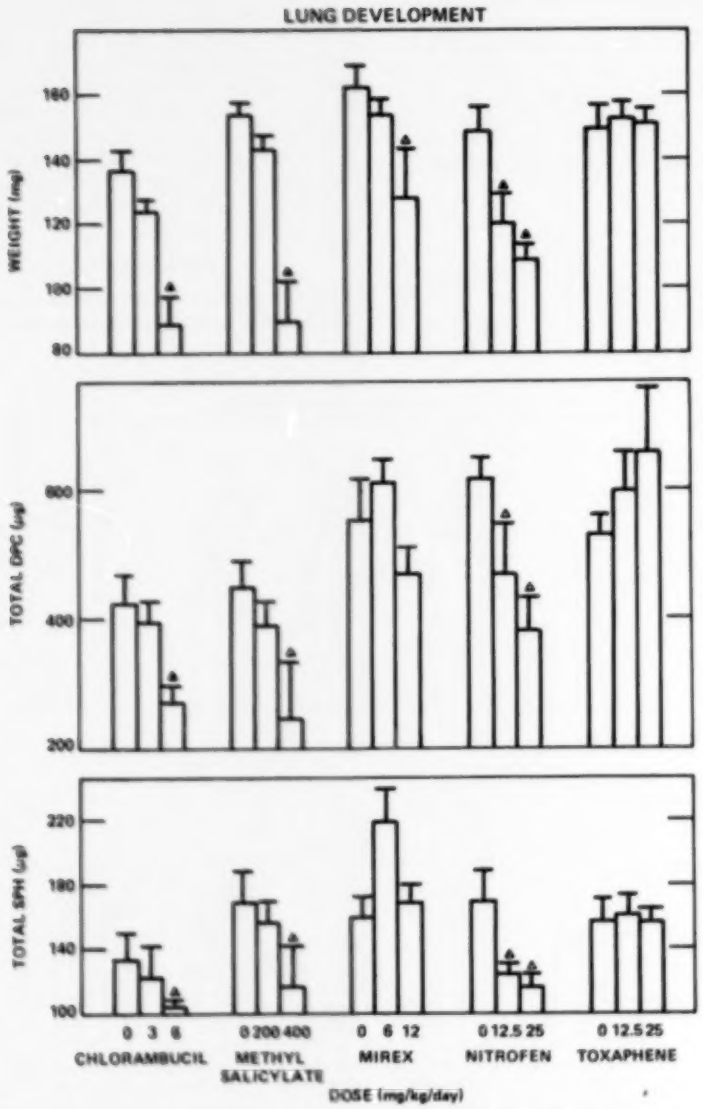

Fig. 3. Effects of five selected compounds on lung development in the 21-day-old fetus. See Figure 2 for details of the presentation. DPC = dipalmitoyl phosphatidylcholine, SPH = sphingomyelin.

Brain development

Dose-related reductions in brain weight occurred following exposure to CHL, MES, MIR, and NIT, while brain protein content was reduced by CHL and MIR and brain DNA by MIR and NIT. Significant overall reductions in the composite brain index were noted in the high dose groups of CHL, MIR, and NIT and ranged upward to a 1.5 day delay for MIR. However, the changes in the composite brain indexes were directly related to growth retardation as covariate anlaysis eliminated the effect in each instance.

Lung development

Dose-related reductions in lung growth were observed for CHL, MES, MIR, and NIT. Of these, only the MIR effect proved attributable to overall growth retardation. Pulmonary dipalmitoyl phosphatidylcholine and sphingomyelin contents were reduced by CHL, MES, and NIT. With the exception of the CHL effect, the reductions in phospholipid content were attributable to overall growth retardation. The composite lung index values were significantly reduced in the high CHL, high MES, and both NIT groups. The magnitude of this delay in lung development ranged from a 0.76-day delay in the low NIT group to a 1.19-day delay in the high MES group. For CHL and NIT, these reductions represented specific organ toxicity, as the effects persisted after covariate analysis.

Liver development

All coumpounds except TOX significantly reduced liver growth in a dose-related fashion.

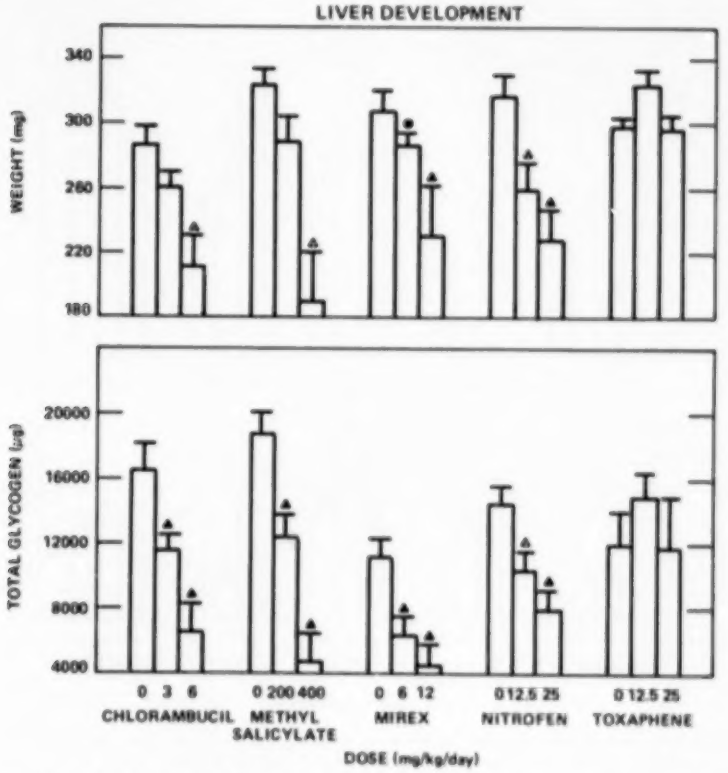

Fig. 4. Effects of five selected compounds on liver development in the 21-day-old fetus. See Figure 2 for details of the presentation.

The reductions in weight persisted after covariate analysis for both MIR groups and for the high NIT group. Drastic reductions (often more than 50%) in hepatic glycogen content occurred in every dose group for those compounds causing reductions in liver weight. Significant reductions in the composite liver index were observed for both dose groups in

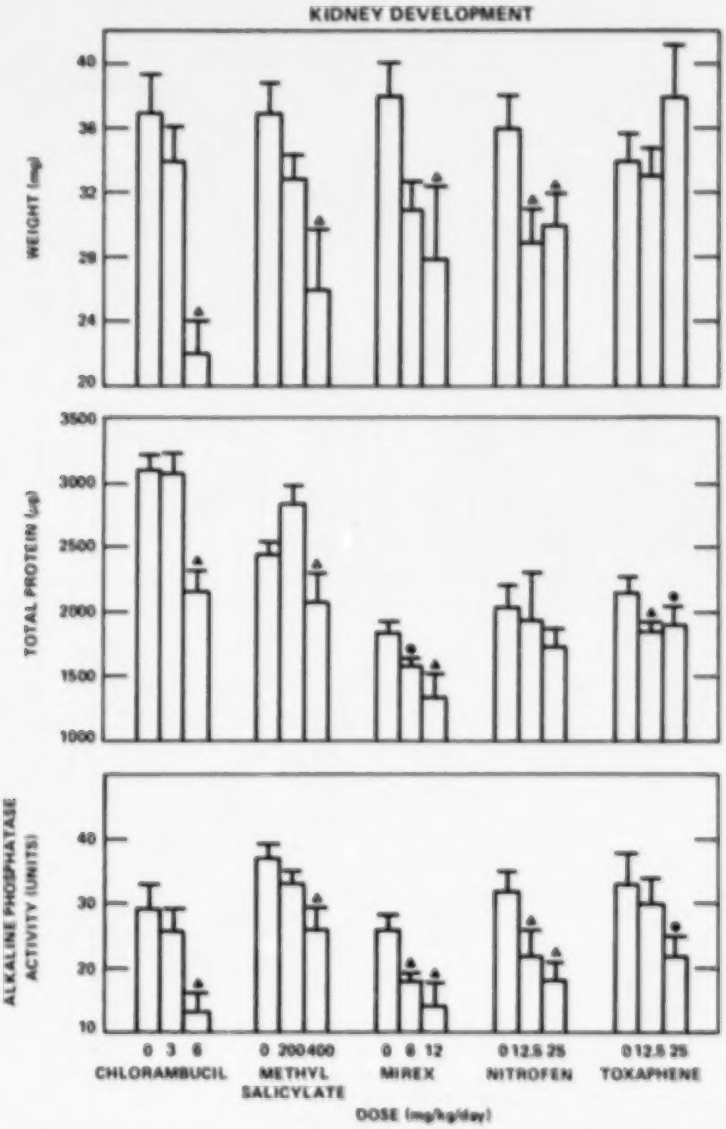

Fig. 5. Effects of five selected compounds on kidney development in the 21-day-old fetus. See Figure 2 for details of the presentation.

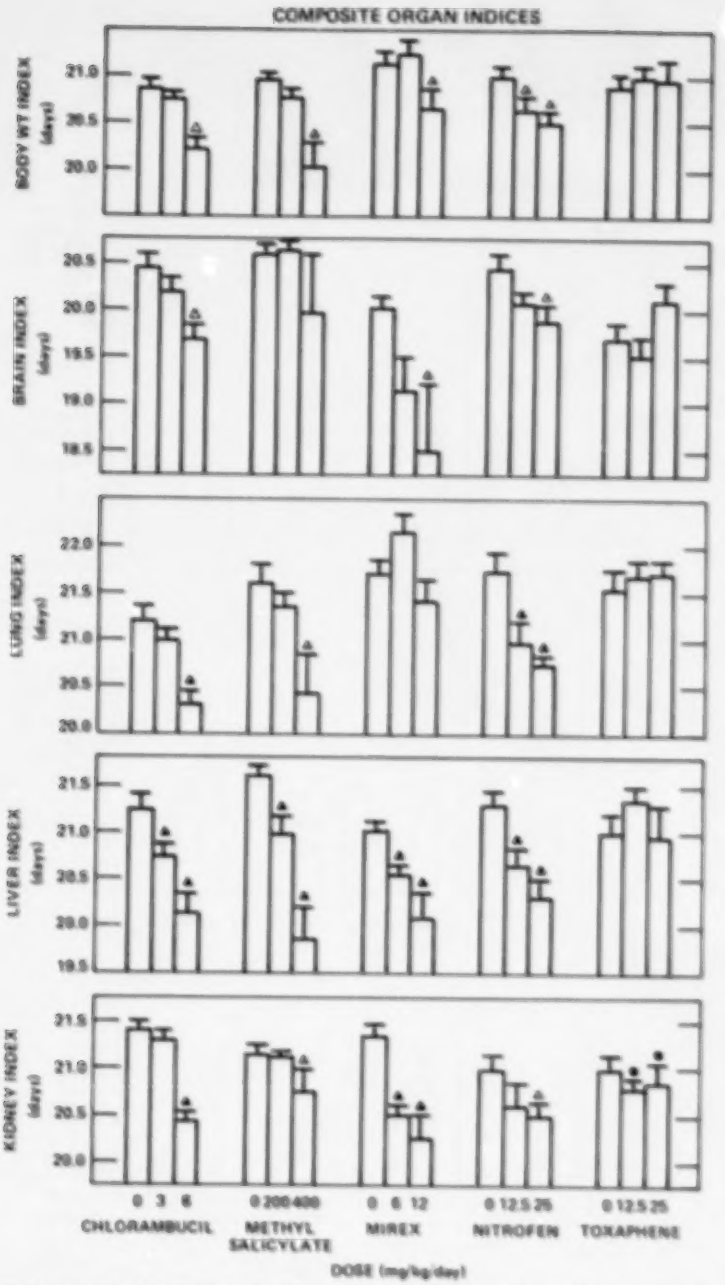

Fig. 6. Composite organ indices for five selected compounds. Data were generated by applying factor analysis for all variables for a particular organ in the normative series. The derived linear weighting coefficients for the same groups of variables were then applied to the experimental series. See Materials and Methods section for more detail. Ordinate values represent the calculated theoretical developmental age (in days) for body weight and organ maturation. Statistics are as described in Figure 2.

every compound except TOC and ranged from a 0.47-day delay in the low MIR group to a 1.76-day delay in the high MES group. In every instance, these reductions represent specific organ toxicity.

Kidney development

Dose-related reductions in kidney weight were observed following exposure to CHL, MES, MIR, and NIT, although all weight reductions were related to overall growth retardation. Reductions in protein content were evident for all compounds except NIT, while all compounds reduced alkaline phosphatase activity in a dose-related manner. The reductions in protein content and alkaline phosphatase activity persisted after correcting for body weight effects for CHL, MIR, and TOX. Interestingly, since the TOX effects occurred in the absence of an overall organ weight effect, the effect grew in significance after covariate analysis. All compounds caused dose-related reduction in the composite kidney index, and these changes represented organ specific toxicity for CHL, MIR, and TOX. Significant retardations in overall renal development ranged from 0.16 days for high TOX group to 0.98 days for the high CHL group.

DISCUSSION

From the data generated in the normative series, it can be observed that examination of organ/body weight ratios on a single day of gestation can provide misleading information as to the nature of a particular response to a putative teratogen. For example, since the relative weight of the brain decreases during late gestation, an overall retardation in the rate of body growth will result in an increase in the relative weight of that organ. The mere fact that the weight of the brain relative to the body weight may be greater in treated groups as compared with that observed in a control group does not imply that the brain is protected from an insult at the expense of the body; rather it represents the normal developmental sequence of brain growth. The real question that must be asked is whether that reduction in growth is totally attributable to overall growth retardation (i.e., does it represent a generalized toxicity?) or is it greater in magnitude than predicted by the overall retardation (i.e., does it represent organ specific toxicity?). Relative lung weights are even more difficult to interpret, as the relative growth of that organ during late development corresponds in shape to an inverted "U." For such reasons, we opted to analyze total organ weights in the experimental series using analysis of covariance procedures to correct for differences due to changes in body weight. This approach allowed us to detect generalized toxicity as well as organ-specific toxicity.

We observed expected patterns of organ biochemical differentiation for the brain (growth through hypertrophy rather than hyperplasia), lungs (a surge in phospholipid accumulation between days 21 and 22), liver (glycogen deposition occurring at an ever increasing rate), and kidney (large increase in alkaline phosphatase activity just prior to term).

Of the five compounds used in the experimental series, four resulted in significant dose-related fetal growth retardation. Significant reductions in the composite organ indices were noted with three compounds for the brain and lungs, four with liver, and with all five for the kidney. All three examples of reduced brain maturation represented generalized toxicity. However, for the other organs examined effects were observed that were not solely attributable to fetal growth retardation. The liver and kidney appeared particularly prone to organ-specific toxicity. For both of these organs, the biochemical variables monitored often provided more conclusive evidence of the toxicity than did organ weights.

In summary, we have demonstrated that the effects of fetal growth retardation resulting from exposure to exogenous agents are not equally distributed among the organs examined. While no particular clustering of organ specific effects were noted in this study, the liver and kidney appeared more sensitive to insult than did the brain and lungs. Future research is now being directed at the sequelae of such effects on the health status of the neonate. Correlations between fetal and neonatal toxicity will then help define the biological significance of "fetotoxicity." Does the diminution of hepatic glycogen deposition foreshadow a hypoglycemic state in the newborn? Does the decreased specific activity of a renal tubular enzyme indicate altered development of the nephron that may lead to renal dysfunction in the neonate? These and other such questions are currently being addressed in our laboratory.

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